

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶: B01D 15/08, C07K 1/26	A1	(11) International Publication Number: WO 95/10344 (43) International Publication Date: 20 April 1995 (20.04.95)
(21) International Application Number: PCT/US94/11430 (22) International Filing Date: 14 October 1994 (14.10.94) (30) Priority Data: 135,971 14 October 1993 (14.10.93) US 322,328 13 October 1994 (13.10.94) US (71) Applicant: SARASEP, INC. [US/US]; Suite #10, 1600 Wyatt Drive, Santa Clara, CA 95054 (US). (72) Inventors: GJERDE, Douglas, T.; 12295 Woodside Drive, Saratoga, CA 95070 (US). YENGOYAN, Leon; 6714 Leyland Park Drive, San Jose, CA 95120 (US). (74) Agents: WALKER, William, B. et al; Oblon, Spivak, McClelland, Maier & Neustadt, P.C., 4th floor, 1755 Jefferson Davis Highway, Arlington, VA 22202 (US).		(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ). Published <i>With international search report.</i>
(54) Title: BEADED CAPILLARIES FOR CAPILLARY ELECTROPHORESIS AND CHROMATOGRAPHIC SEPARATIONS		
(57) Abstract A capillary having beads bound to the inside wall thereof is useful in capillary electrophoresis and capillary liquid chromatography. The beads may be ion exchange resin beads, chelating resin beads, reverse phase beads or beads having affinity sites. The beaded capillary allows control of direction and magnitude of electroosmotic flow through the capillary.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LJ	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

Description

Beaded Capillaries For Capillary Electrophoresis and Chromatographic Separations

Technical Field

The present invention relates to capillary electrophoresis and chromatographic separations. In particular, the invention relates to novel open tubular capillaries having insoluble beads bonded to the inside surface of a capillary.

Background Art

Capillary electrophoresis (CE) is a method for separating liquid components using open tubular capillaries. In CE, high-voltage is used to separate ions on the basis of mobility. Generally, suitable capillaries are made of plastic or fused silica of any length, but ranging from about 10-100 cm and an internal diameter (i.d.) of about 2-300 microns. The capillary is filled with an electrolyte and then the sample is introduced into one end of the capillary by means of siphoning or electrokinetic transport. The tube is then placed in contact with the electrolyte and a voltage potential of about 10-30 kV is applied across the capillary for a typical 100 cm capillary. Analytes in the sample of opposite charge are attracted and travel to the respective electrode. Positively charged ions are attracted to the cathode; negatively charged ions are attracted to the anode. A detection cell is located near the capillary end and the sample ion peaks are measured as they pass across the detection cell, e.g., an ultraviolet (UV) absorption detector.

Ions with the highest mobility travel the fastest through the capillary and are detected first. Ions having progressively lower mobility travel through the cell more slowly and are detected in the order of their relative mobility. Thus, in CE the separation of ions is not based on capillary wall interactions, but rather is based on the relative mobilities of the ions travelling through the

-2-

capillary. Since the sample ions are not intended to interact or partition with the capillary walls, early research in capillary electrophoresis was conducted using fused silica capillaries having unmodified inner walls.

Nevertheless, modification of a CE capillary wall is useful and sometimes necessary. In some applications, analytes must be prevented from adsorbing on the wall of the capillary. If adsorption occurs, the analyte peaks are broadened or may not be separated. Adsorption is a particularly important problem in protein separations. Proteins can be prevented from adsorbing onto capillary walls by treating the capillary walls to produce a charged surface which repels the proteins. See US 4,680,201; Huang et al, J. Microcol. Sep., (1993) 5:199-205; Yao et al, J. Chromatogr., (1993) 636:21.

Another reason for modifying the walls of a capillary is to suppress electroosmotic flow (EOF). EOF results from the flow of the bulk liquid of the electrolyte when a potential is applied across the capillary. For applications involving capillary isoelectric focusing, for example in protein separations, it is necessary to eliminate or substantially reduce the EOF. In capillary isoelectric focusing, a pH gradient is established along the length of the capillary. The capillary is then filled with a sample containing proteins and a potential is applied across the capillary. The proteins migrate to a point in the capillary which corresponds to their individual isoelectric points (the point at which the protein has a zero net charge). Any EOF or bulk flow of electrolyte prevents establishment of a stable pH gradient which, in turn, destroys the separation characteristics and causes diffusion of the proteins. At zero EOF, separation integrity is maintained. When the proteins in the mixture have stabilized at specific points in the capillary corresponding to their isoelectric points, the bulk flow is mobilized to release the proteins by pumping (while maintaining applied voltage), by

-3-

changing the pH gradient or by changing the ionic strength in the buffer. The protein bands are collected sequentially for further workup.

EOF is a phenomenon caused by a stationary or semi-permanent charge on the capillary walls. For example, weak cationic exchange sites (due to ionized silanol groups from the fused silica) have a negative charge. Cations in the electrolyte form a diffusion layer of positive ions attracted to the negative wall charge. When a potential is applied, these cations are attracted to the negatively charged cathode. EOF or flow of the bulk of the electrolyte fluid results from "dragging" of water molecules which hydrate these cations as the cations migrate to the cathode. Thus, a negatively charged capillary wall has an EOF to the cathode. Conversely, a positively charged capillary wall has an EOF to the anode.

Methods are known for suppression of the EOF by eliminating charges on the capillary wall. US 4,608,201 discloses coating the inside wall of a capillary with a neutral polyacrylamide polymer. Monomer and catalyst are added to the capillary and polymerization is initiated inside the capillary to deposit the neutral polymer on the capillary walls. Capillaries having polymer coated inner walls are available commercially (ABI, Foster City, CA). Polyacrylamide coatings have the disadvantage that the coating is not pH stable and must often be replaced.

Finally, another reason for controlling the magnitude and direction of the EOF is to decrease the analysis time. This is accomplished by controlling the EOF so that it is in the same direction as the migration of the analyte ions. As noted above, anions migrate to the anode. Normal EOF in fused silica capillaries is toward the cathode due to the negative charge on the fused silica surface. Anion migration is counter to the EOF flow and CE analysis of anions with this type of capillary requires long times and generally produces unusable

-4-

electropherograms for some ions. However, if a capillary is modified to have a positive wall charge (anion exchange sites), the EOF is toward the anode. In this case, the EOF is in the same direction as anion migration resulting in improved performance and shortened run times. Good results have been obtained with anion exchange groups coated on a capillary wall. The anion exchange groups may be coated on the wall by means of dynamic coating with specific reagents added continuously to the analyte (US 5,015,350). Surfactants containing anion exchange groups have been used to coat capillary walls on the basis of dynamic coating. In dynamic coating, an equilibrium is established between the surfactant in the electrolyte and surfactant transiently bound to the capillary wall. However, due to equilibrium differences, dynamic coating often gives nonreproducible results. Additionally, the equilibrium of the dynamic coating in solution with the wall is readily disturbed by the sample ion strength, pH, etc.

A semi-permanent anion exchange coating has been established by adsorption (J.E. Wiktorowicz and J.C. Colburn, *Electrophoresis*, (1990), 11:769) and by molecular and polymeric binding and coating (R. Keer and L. Jung, French Patent WO 92/05433; J.K. Towns and F.E. Regnier, *J. Chromatogr.* (1990) 516:69; A.M. Dougherty et al, *J. of Liq. Chromatogr.* (1991) 14:907; J.T. Smith and Z. El Rassi, *Electrophoresis*, (1993), 14:396). However, these coatings are unstable and degrade in aqueous buffers particularly at high and low pH. Both static and dynamic coatings are frequently unstable in high organic solvent concentrations.

US 4,486,312 describes a device and method of using tubing with protuberances on its inner wall for liquid chromatography.

US 4,101,460 describes a packed column bead for liquid chromatography in which the beads contain a large polymeric substrate and a small insoluble polymer agglomerated on the

surface of the substrate to produce a packed column for chromatography.

US 5,151,164 describes capillary zone electrophoresis in which an external electric field is applied to the exterior of the capillary tube as a means of controlling the polarity and magnitude of the surface charge on the interior surface of the capillary.

A need continues to exist for a method of coating the inner walls of capillaries for the purpose of modifying the EOF and improving the efficiency of capillary separation techniques.

Disclosure of the Invention

Accordingly, one object of the present invention is to provide a method of bonding ion exchange groups to the inside walls of a capillary tube.

A further object is to provide a method of bonding ion exchange groups to capillary walls which provides substantially stable coatings both at high and low electrolyte pH values.

A further object of the invention is to provide coated capillaries which can be used to modify a sample prior to separation by removing impurities or changing the pH of a sample prior to detection.

These and other objects of the present invention which will become apparent from the following disclosure have been achieved by the beaded capillary of the present invention in which insoluble beads are bonded to the inside capillary walls to produce a "beaded capillary". The beads which are bonded to the inside capillary walls in the present invention are generally spherical. However, the beads need not be perfectly spherical and can have any irregular particle shape so long as the beads can be bonded to the capillary wall. The invention also includes capillary electrophoresis and capillary liquid

-6-

chromatography apparatus containing the capillary of the present invention and the use of the capillary to separate analytes, control EOF and prevent adsorption of analytes on the capillary wall. In addition, the capillary of the present invention may be used to attract analytes to the capillary wall to control selectivity in capillary electrophoresis and capillary ion chromatography. The capillary of the present invention may also be used to preconcentrate samples, trap or remove interfering substances in the sample matrix, and to adjust pH of a sample.

Brief Description of the Drawings

Figure 1 shows an electropherogram for the separation of anions described in Example 2.

Figure 2 shows an electropherogram for the separation of anions described in Example 3.

Best Mode for Carrying Out the Invention

The beaded capillaries of the present invention are prepared by bonding small reagent particles or beads (collectively referred to below as "beads" only) to the inside wall of a capillary tube. Depending on the specific application, the inside diameter of the capillary tube will range from about 2 to about 800 microns, preferably about 2 to about 300 microns, more preferably about 5 to about 75 microns. Such capillary tubes are known in the art and any conventional capillary used for capillary electrophoresis or chromatography (gas or liquid) may be used in the present invention. Typically, the capillaries are made of glass or preferably fused silica. However, capillaries prepared from suitable plastics such as perfluorinated plastics (i.e. polytetrafluoroethylene, PTFE) may also be used. The capillary may have any desired length. Typically, the capillary will have a length from 10 to about 200 centimeters although longer

-7-

columns may also be used. Columns suitable for use in miniaturized chromatography equipment using shorter columns with lengths of about 0.5-5 centimeters may also be used in the present invention. The cross section of the capillary is generally circular, but may also be oval, square, rectangular, etc. These very small capillaries (microcapillaries) can be used in conjunction with known electronic microcircuitry including microcircuits contained on a substrate such as silicon or germanium. The microcapillary may be located on the substrate, etched into the substrate, or may be drilled into the solid substrate itself. In this embodiment, the capillary is in the form of a microbore through the substrate.

The beaded capillaries of the present invention are small open tubes of any length having small insoluble beads attached to the inside wall. The insoluble beads are normally spherical with an average particle size ranging from about 0.005 to about 2 microns, preferably about 0.02 to about 0.3 microns.

Individual bead sizes may vary so long as substantially all of the beads have a diameter less than about 2.0 microns. By "substantially all of the beads" is meant that 95-100%, preferably 98-100% of the beads have a diameter of about 2.0 microns or less. In a particularly preferred embodiment, the bead diameters fall within a narrow diameter range and thereby provide a narrow particle size distribution.

The beads are attached to the capillary wall such that fluid (e.g., electrolyte) flow through the capillary is maintained. Although suitable fluid flow is maintained for a wide range of bead diameters, the ratio of average bead diameter to capillary inside diameter is preferably in the range of about 2.5×10^{-5} to about 2.0×10^{-1} , more preferably in the range of about 1.0×10^{-3} to about 1.5×10^{-2} .

Suitable beads may incorporate chemical functional groups, such as cation exchange resins, anion exchange resins, reversed phase column chromatographic materials, chelating ion exchange

resins, and any other type of bead which is capable of binding the substance of interest.

Many substances have the ability to behave as ion-exchangers. These substances include clays, natural and synthetic zeolites, certain glasses, some inorganic oxides and insoluble salts, and some functionalized organic polymers. The most important type of ion-exchange resins are made from organic polymers such as styrene-divinylbenzene copolymers. Examples of typical ion-exchangers useable in the present invention are shown below. These materials may be milled to the desired particle size using known methods and equipment.

Examples of ion exchange types.

Type	Example	Example Formula
Clay	kaolinite	$\text{Al}_2\text{Si}_2\text{O}_5(\text{OH})_4$
Zeolite	natrolite	$\text{Na}_2(\text{Al}_2\text{Si}_3\text{O}_{10}) \cdot 2\text{H}_2\text{O}$
Inorganic Oxide	alumina	Al_2O_3
Inorganic Salt	zirconium phosphate	$\text{Zr}(\text{HPO}_4)_2 \cdot \text{H}_2\text{O}$
Polymer	DOWEX 1X8	$\text{C}_2\text{H}_3(\text{C}_6\text{H}_4)\text{CH}_2\text{N}(\text{CH}_3)_3\text{Cl}$

Chelating ion-exchange resins have a chelating functional group built into or covalently attached to a polymer resin. Chelating resins are capable of taking up only a small group of metal ions or may complex a larger group of metal ions, where selectivity is obtained through pH control. Typical chelating resins which can be used in the present invention include iminodiacetic acid resins, hydroxyoxime resins, thioglycolate resins, acetamide resins, N-benzoylmethyl-hydroxylamine resins, isothiuronium resins, and resins containing crown ether groups (e.g., 12-crown-4, 18-crown-6) which take up and chelate alkali and alkaline earth metal ions. Specific resins are commercially available in particulate form in a variety of particle sizes. These resins may be milled to the desired particle size using known methods. Any of these

commercially available chelating resins may be used in the present invention.

Additional chelating ion-exchange and ion-exchange resins which can be used as the beads in the present invention are described in D.T. Gjerde and J.S. Fritz, "Ion Chromatography", Huethig:Heidelberg, 1987. Normal phase and reversed phase chromatography materials which can be used as the beads of the present invention are described in L.R. Snyder and J.J. Kirkland, "Introduction to Modern Liquid Chromatography", Wiley:New York, 1979. Any of the resins or chromatography materials described in these references may be used in the present invention and milled to the desired particle size, provided they meet the pH stability requirements necessary for the particular application for which they are intended.

Additionally, the beads may contain affinity sites. Such affinity sites include receptors for the analyte. The receptor which is bound to the bead may be any of the well-known receptors including antibodies which specifically bind the analyte as well as other biological receptors such as binding proteins which are known to bind the analyte of interest. Non-limiting examples of such analyte/binding proteins include thyroxin/thyroxin-binding globulin, cortisol/corticosterone-binding globulin and insulin/insulin-binding proteins. The affinity receptor or antibody can be bound to the bead using known reactions which are well known for binding such receptors to polymeric beads in conventional immunoassays.

The beads may be bonded or attached to the inside wall of the capillary by a variety of means. In one embodiment of the invention, the beads are attached to the capillary walls through a combination of electrostatic and physical forces. A beaded capillary is prepared by pumping an ion-exchange (bead) suspension through the capillary until suspension material exits from the opposite end of the capillary. The ion-exchange beads are attracted and attached to the wall of

-10-

the capillary producing the beaded capillary of the present invention. For example, a beaded, fused silica capillary can be prepared by pumping an anion exchange suspension through a base conditioned capillary. The anion exchange beads are attached to the wall of the capillary.

Preferably, the capillary is thoroughly washed and cleaned before attaching the insoluble beads to the inside wall of the capillary. Organic contaminants can be removed by flushing the capillary with a suitable organic solvent such as tetrahydrofuran (THF). Ionic contaminants can be removed by alternately flushing the capillary with acid and base solutions. Finally, the capillary should be thoroughly washed with water to remove any residual contaminants and remaining acid, base or organic solvent.

Anion exchange sites on the anion exchange beads produce an EOF in the anode direction. This is opposite to the EOF of bare, unmodified silica which has an EOF in the cathode direction as described above. The reverse EOF flow in this capillary is stable at both high and low pH conditions. By "stable" as used with respect to this invention means a beaded capillary in which a substantial amount of the beads remain attached to the capillary wall after washing with 200 column volumes of acid or base eluate. The stability of the beaded capillary can be determined by measuring the EOF flow after 200 column volumes of 1.0 M HCl, saturated NaCl solution, NaOH solution (pH 11.5), or 100% tetrahydrofuran (THF) are passed through the capillary. A reduction in EOF of 20% or less after 200 column volumes of eluate indicates a stable beaded column and therefore a column in which a substantial amount of the beads have remained attached to the capillary wall. The stability is determined by producing similar EOF measurements using mesityl oxide as a neutral marker with a TRIS buffer (pH 8.0).

-11-

The stability of the beaded capillaries of the present invention at very low pH (acid, $\text{pH} < 1.0$) and very high pH (base, $\text{pH} > 12.5$) is surprising especially considering all previous work performed with silica-based HPLC packings. As the pH of the eluate is lowered, one would expect the silanol groups to become protonated with subsequent release of the beads from the capillary wall as the electrostatic attraction is decreased. However, it has been discovered that the beads are stable on the capillary wall even when the capillary is treated with eluate at a very acid pH, much lower than the pK_a of the silanol groups. While not being bound by any particular theory, it is believed that the stability of the beaded capillary of the invention is due at least in part to shielding of the silanol groups from acid or base reagents by the beads which are attached to the capillary surface.

In addition to unmodified fused silica capillaries, cation exchange sites may be attached to the capillary wall of capillaries containing strong acid surface groups. The strong acid groups produce a stronger electrostatic bond because the strong acid sites on the capillary wall generate stronger electrostatic attraction to the ion exchange bead. For example, strong acid groups on a capillary prepared from a perfluorinated substrate or a capillary having strong acid binding sites on the capillary wall may be used to attach anion exchange beads to the capillary wall. In the same manner as described above, a suspension of anion exchange beads are passed through the capillary and attached to the surface of the capillary wall. A suitable strong acid-containing capillary is commercially available under the trade name NAFION (Dow Chemical Company).

When the beads contain ion exchange sites, the beaded capillaries of the present invention may have a single layer of beads attached to the capillary wall or multiple alternate layers of ion-exchange beads. A first layer of ion-exchange

-12-

beads is prepared on the capillary wall by passing a suspension of anion exchange beads through the capillary resulting in attachment of ion-exchange beads to the capillary wall and producing an anion exchange beaded capillary. A second layer of beads can be formed on this first layer by passing a suspension of cation exchange material through the anion exchange beaded capillary resulting in attachment and attraction of the cation exchange beads to the first layer. Thus, a double layer beaded capillary is formed where the top layer is cation exchange beads and the bottom layer is anion exchange beads. The EOF of this double-layer beaded capillary is the reverse of the EOF of the single layer anion exchange beaded capillary; the EOF of the double-layer capillary is now toward the cathode. This method can be used to form a beaded capillary having multiple alternating layers of ion exchange beads. Each time a new layer is added, the direction of the EOF flow is characteristic of the net charge which is exposed by the top layer of beads. The beads remain attracted to the capillary wall by electrostatic and physical forces producing stable beaded capillaries.

The synthesis of beaded capillaries having multiple layers of beads allows the preparation of beaded capillaries having simultaneously exposed anion and cation exchange sites. That is, the layers of beads on the capillary are uniform but do not completely cover the surface of the capillary wall or the prior layer of attached beads. The net charge of the exposed ion exchange sites determines the direction of EOF flow when both cation and anion sites are exposed.

High surface area, low capacity macroporous beads attached to the capillary wall significantly increase the surface area and impart partitioning or adsorptive capacity to the capillary wall. Suitable high surface area low capacity macroporous beads include small beads based on the chemistry used for anion exchangers in ion chromatography (D.T. Gjerd and J.S. Fritz,

"Ion Chromatography", Huethig:Heidelberg, 1987). For example, a 400 m²/G macroporous anion exchange bead with low capacity groups (about 0.1 mequiv/g) can be attached to the surface of a capillary producing a high surface area capillary wall.

Chelating ion exchange beads are also ion exchange beads and are attached to the capillary wall in a manner analogous to the way in which cation and anion exchange beads are attached to the capillary wall as described above.

The beaded capillaries have many uses. The attachment of anion, cation, and chelating ion exchange sites to the capillary wall modifies the properties of the capillary allowing control of the direction and magnitude of the EOF in capillary electrophoresis applications. The bead attached to the capillary wall is generally spherical and ion exchange sites (charges) are immobilized on the capillary wall in all directions on the spherical particle. When negative charges are attached to the capillary wall (cation exchange resin) the EOF is toward the cathode. When positive charges are attached to the capillary wall (anion exchange resin) the EOF is toward the anode. The magnitude of the EOF is proportional to the strength of the charges on the attached beads. For example, at low pH strong cation exchange sites will produce a stronger cathodal EOF than weak cation exchange sites. The present invention, therefore, allows one to control both the direction and magnitude of the EOF for capillary electrophoresis applications.

The use of organic solvents with the beaded capillary of the invention allows one to further control separation of ions. The EOF results from dragging of water molecules which hydrate ions as the ions migrate to the anode or cathode. Reducing the hydration diameter of an ion will therefore increase the mobility of the ion in the capillary. The use of organic solvents dehydrates ions reducing their hydration diameters. Suitable organic solvents for use with the beaded capillaries

-14-

of the present invention include C_{1-4} alcohols, or other water-miscible solvents. These organic solvents are added to the electrolyte at a concentration of about 10-90 vol.%.

In addition to controlling EOF, the presence of charged sites on the capillary wall prevents the adsorption of analytes to the wall in electrophoresis applications with relatively large capillaries (about 75-200 microns i.d.). Since separations in CE are based on relative mobilities of ions, it is important to prevent adsorption of the analyte to the capillary wall. Charged walls prevent adsorption of analytes when the charge on the wall is the same as the net charge of the analyte. For example, adsorption of proteins having a net overall positive charge is prevented when the capillary wall has a net positive charge.

The beaded capillaries of the present invention are also useful to separate analytes by capillary electrophoresis and by ion chromatography. Capillaries used for capillary electrophoresis and ion chromatography using an electrokinetic pump to introduce the sample are preferably made of a material which is non-electrically conducting. Ion chromatography using a pressure pump may use either a conducting or non-conducting capillary. A preferred eluate pH for these applications is about pH 10-13. Further, the capillaries should be flexible to allow for adequate sample introduction into the capillary. Suitable non-conducting flexible capillaries are made of fused silica and polymers such as PTFE. A sample of analyte is introduced into one end of the capillary and components of the sample are separated by their relative mobility through the capillary as described above. Bands of the separated analytes are detected as they exit the opposite end of the capillary using a conventional detector for the analyte, for example, a UV absorption detector. The presence of ion exchange beads on the capillary wall influences the EOF and adsorption of analytes as discussed above.

-15-

As the inside diameter of the capillary is decreased, interactions between the analyte passing through the capillary and the beads attached to the capillary wall increase. The beads attract or partition with the analyte to produce a separation which is based on a capillary ion chromatography or an open tubular liquid chromatography (OTLC) separation mechanism. In OTLC, eluate flow through the capillary is maintained by a pressure pump or an electrokinetic pump (bulk fluid flow based on EOF). Generally, OTLC applications require capillary inside diameters of 20 microns or less. A larger capillary inside diameter has poor kinetic transport properties and poor partitioning between the mobile phase and the stationary phase. The use of large capillaries in conventional OTLC results in broadening of chromatographic peaks.

In contrast to conventional OTLC, separations using beaded capillaries having an i.d. of about 20-75 microns are believed to be able to operate by a combination of CE and OTLC separation mechanisms allowing the use of larger capillary inside diameters. Notwithstanding the use of larger capillary diameters, the beaded capillaries of the present invention produce substantially less peak broadening than capillaries of the same size used with conventional OTLC. The beaded capillaries of the invention allow one to separate analytes using larger capillaries while combining the benefits of both CE and OTLC separation mechanisms.

The presence of charged beads on the capillary surface allows one to control the separation efficiency of a capillary in CE for tubes of intermediate i.d., about 20-75 microns. As noted above, it is generally desirable to minimize interactions between the analyte and the capillary wall in CE since separation is based on mobility and not partitioning between the analyte and a stationary phase. However, the beaded capillary of the invention allows one to use a combination of OTLC and CE to effect separation and uses analyte/wall

-16-

interactions to control selectivity. As the capillary i.d. decreases below 20 microns, separation is believed to occur substantially by the OTLC mechanism alone.

The beaded capillaries of the present invention may also be used to preconcentrate an analyte prior to separation or detection. For example, a small short capillary containing cation and anion exchange sites (beads) exposed on the capillary wall may be used to concentrate all charged analytes in a sample prior to separation. The cation exchange sites may be present on a strong acid cation exchanger in the H^+ form. The anion exchange sites may be present on a strong base anion exchange resin in the OH^- form. Filling the capillary with sample results in reversible binding of both anions and cations with release of hydroxide (OH^-) and protons (H^+ ,) respectively, which combine to form water. A capillary would thus pick up and bind all ions in the sample to the capillary walls. The reversibly bound analytes may then be eluted into a separation capillary for CE separation and analysis. Separation begins when the sample is released from the preconcentrator capillary with a carrier electrolyte (buffer). Similarly, a preconcentrator capillary can be used to preconcentrate ionic analytes prior to ion chromatography. Preconcentrator capillaries can be used with a variety of analytical instruments including atomic emission, atomic adsorption and mass spectrometers.

The beaded capillaries of the present invention may also be used to remove interfering substances or to contribute reagents to an analyte solution to facilitate detection. Chelating groups attached to the capillary wall will remove ionic contaminants prior to analysis. For example, a chelating group specific for iron ions will remove iron ions from an analyte solution without interfering with other analytes prior to conventional analysis. Beaded capillaries may also be used to remove interferences in gas chromatography (GC) using

-17-

capillary separation. For example, a beaded capillary containing cation exchange groups in the acid form may be used as a pre-column in the injector port of a gas chromatograph. The cation exchange sites in the beaded capillary bind and trap amine compounds before they can enter a separation capillary.

The beaded capillaries may also be used to modify the properties of analyte solution passing through the capillary prior to analysis. For example, cation exchange beads in the acid form on the capillary wall will contribute buffering protons to the analyte solution to control pH prior to the analysis. Similarly, anion exchange beads in the hydroxide form will contribute buffering hydroxide ions to the analyte solution.

In a further embodiment of the invention, the beads are covalently bound to the capillary wall. When the capillary is a fused silica capillary, covalent bonds may be formed with exposed silanol groups using conventional silane chemistry. For example, the surface of a fused silica capillary can be modified by contact with γ -glycidoxypyrpyltrimethoxysilane and heating. This produces a first layer having reactive glycidoxy groups available for bonding to appropriate nucleophilic groups on the beads used to form the beaded capillary. Reactive groups on the beads include amine, hydroxyl and thiol groups which react with the exposed glycidoxy groups on the first coating layer. Similar reactions have been used to attach hydroxypropyl cellulose to capillary surfaces. See J.T. Smith and Z. El Rassi, *Electrophoresis*, (1993), 14:396-406. These reactions can be used to attach the beads having ion exchange groups, chelating groups, reverse phase groups, and affinity groups.

Other features of this invention will become apparent from the following description of exemplary embodiments of the invention which are given for purposes of illustration and are not intended to be limiting thereof. Examples reduced to

-18-

practice in the laboratory are presented in the past tense; examples constructively reduced to practice by filing are presented in the present tense.

EXAMPLES

Example 1 - Preparation of Beaded Anion Exchanger Capillary

A fused silica capillary (Polymicro, Phoenix, AZ), 75 μ m i.d. (inside diameter), 357 o.d. (outside diameter), L = 60 cm, l = 42 cm, was treated to form a positively charged wall coating. The capillary was conditioned with tetrahydrofuran for 20 minutes at a flow rate of approximately 1 microliter/min, followed by a quick water wash, a 30 minute wash of 1 M NaOH, a quick water wash until neutral, a five minute wash with 1 M HCl, and finally a water wash until neutral. Then the capillary was treated with 20 mM, pH 8.0 TRIS chloride buffer. Finally, the capillary was coated with a dilute suspension, approximately 0.01% wt/vol of 0.1-0.2 micron particle, strong base anion exchanger (acrylic-styrene copolymer with quaternary amine groups, Type A1, Sarasep, Santa Clara, CA). The suspension was passed through the column (approximately 5 minutes) until excess material exited from the end of the capillary. Then, the capillary was washed with the TRIS chloride buffer until the absorbance at 254 nm was constant.

After treatment, the capillary electroosmotic flow was anodal 2.69×10^{-4} cm²/volt-sec using mesityl oxide as the neutral marker in a pH 8.0 TRIS chloride buffer.

A second treatment with the Type A1 anion exchanger suspension resulted in an identical EOF, anodal 2.63×10^{-4} cm²/volt-sec. Then the capillary was treated with 1 mM cetyltrimethylammonium bromide. This treatment also, did not change the EOF (2.90×10^{-4} cm²/volt-sec). The results showed the coverage of the capillary wall was complete with the original treatment of Type A1 anion exchange suspension beads.

-19-

In a second experiment, a second capillary was prepared and the EOF measured as a function of pH in a phosphate buffer. The EOF was constant over a pH range of 6.5 to 11.5 with EOF flows of 2.0×10^{-4} to 1.8×10^{-4} cm²/volt-sec, respectively.

The capillary was then tested for organic solvent stability. The capillary was washed with at least 200 column volumes of tetrahydrofuran. The EOF of the capillary was identical before and after treatment with the solvent.

The capillary was also tested for stability with high concentrations of salts. Two hundred (200) column volumes of a saturated solution of NaCl was pumped through the capillary. After the treatment, the capillary was filled with electrolyte and the EOF measured. There was no change in the EOF.

Next, the capillary was tested for pH stability. The capillary was treated with at least 200 column volumes of pH 2.5, pH 11.5 and then pH 1.5 phosphate buffer. The EOF was measured between each treatment and found to be constant with the original EOF measurements. Then, the capillary was treated with 200 column volumes of 1 M HCl. The EOF did not change with the acid treatment. Finally, the capillary was treated with 200 column volumes pH 12.4 phosphate buffer. After this treatment, the EOF decreased by approximately 20% indicating either a reduction of the positive charges on the capillary wall or an increase of negative charges due to exposed silanol groups.

Example 2 - Use of Beaded Anion Exchange Capillary for Coflow CE Separation of Anions

The capillary described in Example 1 was used to determine the purity of ribonuclease (RNAase). RNAase, 2mg/mL, was separated with a 10 mM sodium phosphate electrolyte buffer at pH 7.3. Direct detection was used at 220 nm and 0.005 AUFS (Absorbance Units Full Scale sensitivity) and 25 kV potential. The major peak component was detected at 6.1 min with an

-20-

impurity at 5.3 min. The impurity was tentatively determined to be RNAase with improper sulfide linkages or an aggregated form. It was present at approximately 5% impurity level.

In another separation, the capillary described in Example 1 was used to separate a mixture of bromide (4 ppm), chloride (2 ppm), sulfate (4 ppm), nitrite (4 ppm), nitrate (4 ppm), fluoride (1 ppm), and phosphate (4 ppm). 5.0 mM sodium chromate (no pH adjustment) was used as the electrolyte and the absorber for indirect detection. There were no other additives in the electrolyte. The separation performed at 15 kV potential was quite rapid (see Fig. 1). These conditions may be used for several types of sample mixtures.

Example 3 - Use of Anion Exchanger Coating for Selectivity Control Using Combination of CE Mobility and OTLC Ion Exchange Partitioning Separation Mechanisms.

A capillary was prepared in exactly the same was as described in Example 1 except that a different anion exchanger (acrylic-styrene copolymer with quaternary and tertiary amine groups, Type A2, Sarasep, Inc.) was used as the wall beads. The same conditions as described in Example 2 was used to separate the mixture of anions. The results are shown in Fig. 2. The degree of separation can be controlled by choosing the type of anion exchanger and capillary as shown in Example 4.

Example 4 - Increased Contribution of OTLC Partitioning Mechanism for CE Separation of Ions.

In Examples 2 and 3 the effect of the OTLC wall partitioning effect is minimal and CE mobility is the major contributor to the separation mechanism. However, the effect of OTLC ion exchange partitioning can be made to have a larger influence by reducing the inside diameter of the capillary in the range of 25-50 microns. This reduces the distance the analytes have to travel to the wall to partition and interact

-21-

with the wall. A 25 micron capillary is made into a beaded anion exchanger capillary according to the procedure stated in Example 1. A mixture of anions is injected into the capillary and separated according to the conditions described in Examples 2 and 3. In this case, ion exchange effects are more pronounced although the separation due to ion mobility is still effective. The elution order of the ions is nitrite, nitrate, sulfate, and then fluoride. In this case, sulfate elutes later than nitrite and nitrate even though sulfate has a higher mobility than these ions. However, fluoride still elutes after sulfate because it has a low mobility relative to the other ions.

Example 5 - Use of Anion Exchanger Capillary for OTLC Ion Exchange Separation of Anions.

At capillary diameters, about 25 microns and smaller, the main contribution to selectivity is due to ion exchange partitioning. A 10 micron capillary is coated with an anion exchanger stationary phase according to the procedure described in Example 1. An eluant of 20 mM sodium hydroxide is pumped through the column, and suppressed conductivity detection is used. The pump may be electrokinetic type (using the electroosmotic flow to pump fluid through the column and detector) or a pressure type pump. A mixture of anions is injected. They partition with the stationary phase on the wall of the capillary and elute in order of: fluoride, chloride, nitrite, bromide, phosphate, and sulfate. The selectivity is consistent with anion exchanger selectivity coefficients of fluoride < chloride < nitrite < bromide < phosphate < sulfate.

Example 6 - Use of a Beaded Capillary to Reduce Electroosmotic Flow.

A beaded capillary is prepared according to the procedure described in Example 1. Then, a dilute solution of C18

-22-

sulfonic acid surfactant salt is pumped through the capillary and washed with buffer. The anion of sulfonic acid forms an ion pair with all of the exposed anion exchange sites. This causes the wall to have a neutral charge and decrease the electroosmotic flow. The capillary is then filled with an electrolyte. The electrolyte contains anions such as phosphate that have much lower selectivity and do not displace the C18 sulfonic acid anions.

Example 7 - Use of Beaded Anion Exchanger Capillary for Preconcentration of Anions.

A beaded anion exchanger capillary is made according to Example 1 described above. A short length (approximately 5 cm) of the capillary is used and treated with 10 mM NaOH. This converts all of the anions exchange sites to the OH-form. The capillary is then filled with the sample. Anions in the sample have higher selectivity for the anion exchanger and attach to the wall releasing hydroxide. The process takes approximately 15 minutes to come to equilibrium. Then, the capillary is attached to an analytical instrument. A buffer is passed through the capillary releasing the anions and the anions are transferred into the instrument or separation column for analysis. The buffer is of sufficient concentration and selectivity (e.g. 100 mM phosphate) so that the anions released travel as a plug to the instrument. Thus, preconcentration factors of greater than 100 can be achieved. Instruments suitable for anion analysis include, mass spectrometer, emission spectrometer, capillary electrophoresis, and liquid chromatograph.

Example 8 - Preparation of a Two Layer and Multi Layered Beaded Capillaries.

A capillary was prepared as described in Example 1. Then a dilute (approximately 0.01% wt/vol.) suspension of Type C1

-23-

fully sulfonated cation exchanger beads in pH 8.0 20 mM TRIS chloride buffer was pumped through the capillary for about 5 minutes and was finally rinsed with 20 mM TRIS chloride (pH 8.0) until the absorbance at 240 nm was near zero. Type C1 beads are a 0.2 micron, full sulfonated poly(styrene/divinylbenzene) based strong acid cation exchanger (Sarasep, Santa Clara, CA). The EOF was measured and then the capillary was treated with a suspension of opposite charge (Type A1 anion exchanger). Each treatment resulted in an additional layer of beads. Shown below is the EOF after each treatment of suspension beads:

Experiment	Ion Exchanger Treatment	EOF Direction	EOF (10^{-4} cm ² /volt-sec)
1	anion-A1	anodal	3.0
2	cation-C1	cathodal	3.0
3	anion-A1	anodal	2.2
4	cation-C1	cathodal	1.6
5	anion-A1	anodal	2.3

Each treatment with the alternate ion exchanger bead reversed the EOF. In this case, the magnitude of the EOF reversal was smaller with each treatment indicating that some of the opposite charge bead was still exposed on the layer just beneath the top layer. More complete coverage is accomplished by using a top bead that has a smaller diameter than the base layer. In this example, both the anion exchanger and cation exchanger beads had similar diameters - approximately 0.1-0.2 microns.

Example 9 - Use of a Two Layer Anion Exchanger/Cation Exchanger Beaded Capillary for Preconcentration.

A short capillary is prepared according to experiment 2 described in Example 8 with the following changes. The anion exchanger coated in the first step is in the OH-form and the cation exchanger is coated in the second step is the H-form.

-24-

The cation suspension does not contain any residual anions that might convert any of the anion exchange sites to another form. Then a sample is used to fill the capillary. The anions from the sample replace the anion exchanger hydroxide anions and cations from the sample replace the cation exchanger hydronium ions. The released hydroxide and hydronium ions combine to form water. This drives the equilibrium so that all of the anions from the sample are taken up by the wall sites. The capillary is then treated to release and analyze the preconcentrated ions as described in Example 7.

Example 10 - Preparation of a Single Layer Cation Exchanger Beaded Capillary.

A capillary containing anion exchange sites is treated with a suspension of Type C1 (Sarasep, Santa Clara, CA) fully sulfonated cation exchanger to form a capillary that contains cation exchange sites on the wall. An anion exchanger capillary contains anion exchange sites that are either part of the capillary wall or bonded to the capillary wall. In this example, tubular polytetrafluoroethene is filled with vinylbenzylchloride monomer and allowed to stand for 1 hour. Then the excess monomer is flushed from the tube with air. The tube is irradiated with gamma radiation to polymerize the monomer. Then the capillary is treated with 10% dimethylethanol amine at 80°C to convert the surface of the capillary wall to strong base anion exchange sites. Finally, the capillary is treated with a suspension of cation exchanger as described in experiment 2 in Example 8. The EOF for the cation exchanger beaded capillary is cathodal. Since the groups are strong acid cation exchange sites, the EOF is not dependent on electrolyte pH.

Example 11 - Use of a Beaded Cation Exchanger for Trapping Amines Prior to GC Separation.

-25-

A cation exchanger capillary sleeve is prepared using procedure in experiment 2 in Example 8. The capillary is treated with 0.1 M nitric acid so that it is in the H-form. The capillary has dimensions - about 300 microns i.d. - so that it is inserted in the injection port of a capillary gas chromatograph (GC) and can interface directly with the bonded phase fused silica gas chromatography column. Samples that are injected in gas chromatograph are vaporized inside the capillary sleeve. Amines and metal contaminants are trapped by the cation exchange sites on the beaded capillary sleeve wall before they can enter the gas chromatography separation column.

Example 12 - Use of a Beaded Capillary to Trap Interference Metals Prior to Detection.

A cation exchanger beaded capillary is prepared according to Example 10. The capillary is connected to a Direct Injection Nebulizer (Cetac Technologies, Omaha, NE) which in turn is connected to a Inductively Coupled Plasma and Mass Spectrometer (ICP-MS). The cation exchanger beaded capillary transports sample from the sample vial to the nebulizer. Cations in the sample [Fe(III) in particular] are taken up by the beaded capillary. Anions such as arsenate, arsenite, selenate, and selenite pass through the capillary unretained and are detected by the ICP-MS. Then, the capillary is treated with 4 N nitric acid to release the cations. The cations travel as a plug into the nebulizer and ICP-MS where they are detected. Then, the capillary is washed with water to make it ready for the next sample.

Example 13 - Effect of Organic Solvents on EOF with Beaded Capillaries.

A beaded anion exchanger is prepared according to Example 1. A separation of anions is performed according to Example

-26-

2. Methanol is added to the electrolyte at a concentration of about 20-80 vol.%. All other conditions are kept constant. Anions that have large hydration diameters are "dehydrated" with the addition of the organic solvent. Thus, anions that have large hydration spheres have increased mobility relative to anions that smaller hydration spheres. Fluoride has increased mobility relative to chloride, and the resolution of fluoride and chloride ion pairs is reduced with the addition of methanol to the electrolyte.

Example 14 - Use of Beaded Capillaries to Perform Reverse Phase OTLC Separations.

A lightly sulfonated macroporous (0.1 mequiv/g) 0.2 um bead suspension is prepared and a 20 um i.d. beaded cation exchanger capillary is prepared according to the procedure described in Example 10. An eluant of 80/20 methanol/water is used to separate a mixture of phenol, benzaldehyde, toluene and ethylbenzene. A pressure pump is used to force the eluant through the open tubular column and 254 nm UV detection. The organic material is separated in the order of phenol, benzaldehyde, toluene and ethylbenzene.

Example 15 - Preparation and Use of Beaded Capillary Containing Affinity Site.

A bare silica capillary can be bonded with anion exchange beads containing an additional organic functional group. As an example the anionic beads may also have aldehyde groups. This functional group binds to amine groups found in biological molecules such as proteins, enzymes, antigens and antibodies as well as many small molecules or ligands. A doubly coated capillary surface will have cathodal flow as in Example 9. These cationic beads may also have additional organic functional groups which can bind to small ligands and biological molecules.

-27-

Anion exchange beads having an aldehyde group are coated on the capillary wall as in Example 1. The capillary is washed with 20 mM TRIS chloride buffer (pH = 8.0). A dilute solution of the amine containing molecule is pumped through the capillary under pH conditions which allow bond formation (a Schiff base). The capillary is then washed free of any unbound ligand and is ready to use. This surface acts as a biospecific site for selective binding of the analytes as they move through the capillary under anodal or cathodal electrophoretic conditions. To maximize the binding of large molecules such as proteins, nucleic acids, and carbohydrates to the beaded surface, the organic functional group may be placed at the end of a linker arm which is part of the beads. This prevents steric exclusion of these large molecules to the surface during electrophoresis.

Obviously , numerous modifications and variations of the present invention are possible in light of the above teachings. It is therefore to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described herein.

Claims

1. A capillary for separating an analyte from a solution containing the analyte, comprising a capillary having insoluble beads attached to the inside wall thereof, said beads having a binding site for said analyte, and said insoluble beads remaining attached to said inside wall in the pH range 0-12.5.

2. The capillary of Claim 1, wherein said beads have ion exchange binding sites.

3. The capillary of Claim 2, wherein said ion exchange binding sites are cation exchange binding sites.

4. The capillary of Claim 2, wherein said ion exchange binding sites are anion exchange binding sites.

5. The capillary of Claim 2, wherein said ion exchange binding sites are chelating ion exchange binding sites.

6. The capillary of Claim 1, wherein said beads have reversed phase binding sites.

7. The capillary of Claim 1, wherein said beads have affinity binding sites.

8. The capillary of Claim 1, having an inside diameter of about 2 to about 800 microns.

9. The capillary of Claim 8, having an inside diameter of about 5 to about 75 microns.

10. The capillary of Claim 1, wherein said capillary is a fused silica capillary.

11. The capillary of Claim 1, wherein said insoluble beads have an average diameter of about 0.005 to about 2 microns.

12. The capillary of Claim 11, wherein said insoluble beads have an average diameter of about 0.02 to about 0.3 microns.

13. The capillary of Claim 2, having a plurality of layers of insoluble beads, said plurality of layers containing alternating layers of cation and anion exchange beads.

-29-

14. The capillary of Claim 1, wherein said capillary is within a solid substrate.

15. The capillary of Claim 1, wherein said capillary is non-electrically conducting.

16. A method of analyzing a fluid sample, comprising the steps of:

flowing a fluid sample containing analytes through an open tubular capillary having insoluble beads attached to the inside wall thereof, said beads having at least one binding site for an analyte, wherein said analyte is bound to said binding site; eluting said analyte from said capillary; and detecting said eluted analyte.

17. The method of Claim 16, wherein said method is capillary electrophoresis.

18. The method of Claim 16, wherein said method is open tubular liquid chromatography.

19. The method of Claim 16, wherein said method is capillary electrophoresis in combination with open tubular liquid chromatography.

20. The method of Claim 16, wherein said method is ion chromatography.

21. The method of Claim 16, wherein said method is ion chromatography in combination with capillary electrophoresis.

22. The method of Claim 16, wherein said eluting step comprises elution with an eluant comprising an organic solvent.

23. The method of Claim 16, wherein said fluid sample contains a contaminating analyte, and said contaminating analyte is bound to said binding site thereby removing said contaminating analyte from said fluid sample.

24. A method of increasing or decreasing the pH of a fluid sample, comprising the step of:

flowing said fluid sample through an open tubular capillary having insoluble beads attached to the inside wall thereof, said beads having ion exchange sites in the proton or

hydroxide form, wherein protons or hydroxide ions are released from said ion exchange sites to said sample during said flowing step to decrease or increase the pH of said fluid sample, respectively.

25. A method of concentrating an analyte in a fluid sample, comprising the steps of:

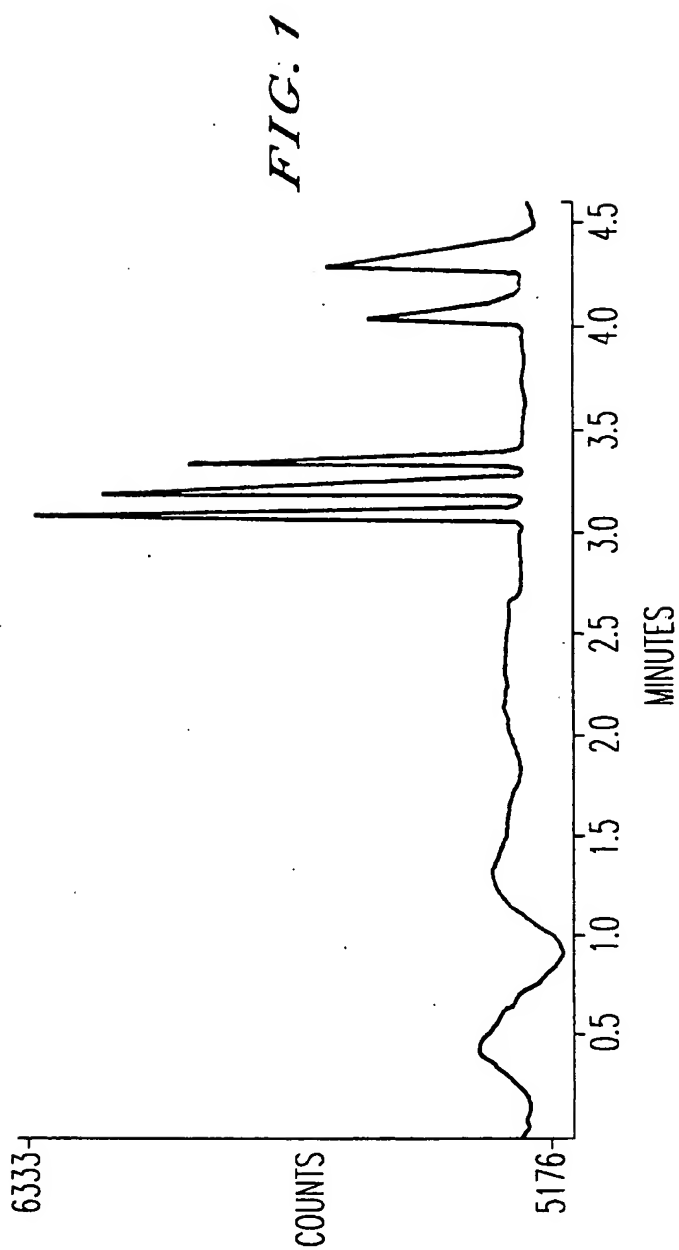
flowing a fluid sample containing an analyte through an open tubular capillary having insoluble beads attached to the inside wall thereof, said beads having a binding site for said analyte;

eluting said bound analyte from said capillary with an eluant, wherein the volume of said eluant is less than the volume of said fluid sample, thereby producing a concentrated solution of analyte.

26. A method of preparing an open tubular capillary having insoluble beads attached to the inside wall thereof, comprising the step of:

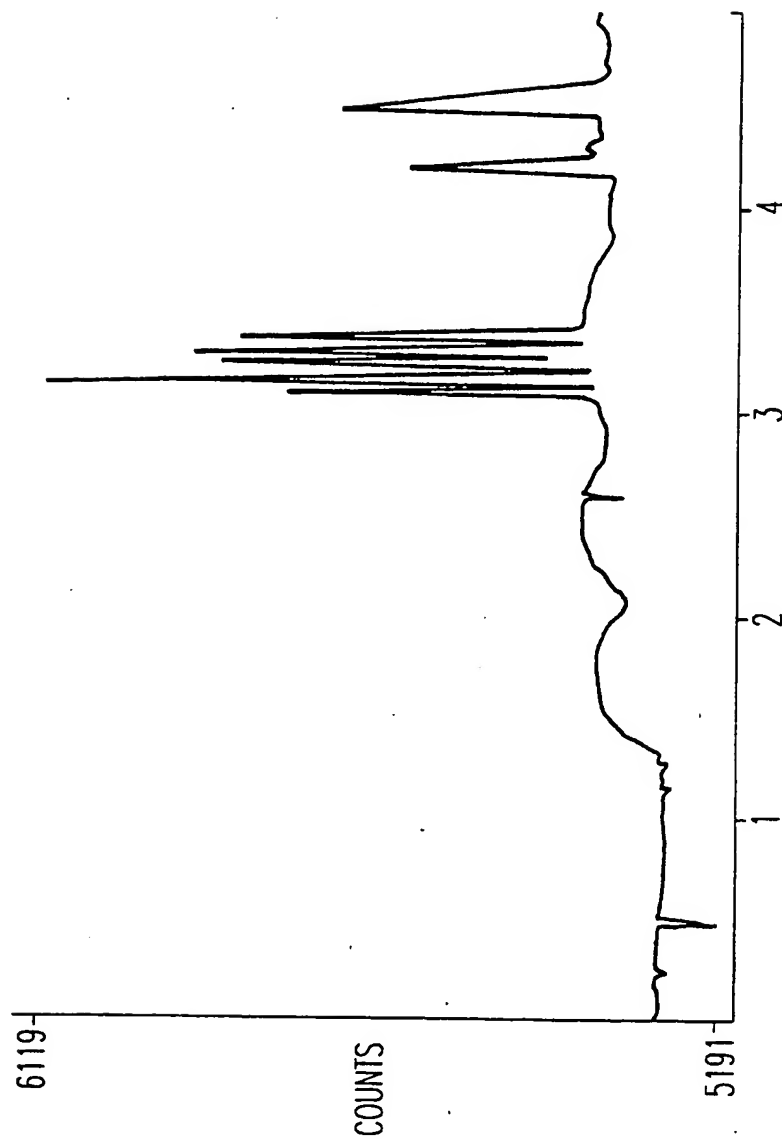
flowing a fluid suspension of insoluble beads through an open tubular capillary having charge sites on the walls thereof, whereby said beads bind to the inside wall of said capillary.

1/2



2/2

FIG. 2



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/11430

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :B01D 15/08; C07K 1/26

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 73/61.53; 204/180.1, 183.1-3, 299R, 302; 210/198.2; 422/59, 60, 70, 101; 436/161, 527, 531, 534

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US, A, 3,488,922 (KIRKLAND) 13 January 1970, col. 5, lines 29-31.	1-16, 18, 20, 22-23, 25-26 ----- 24
Y	US, A, 5,028,415 (BENEDICT ET AL.) 02 July 1991, col. 5, lines 60-61.	24
A	US, A, 5,143,753 (NOVOTNY ET AL.) 01 September 1992, see entire document.	1-26



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	
A document defining the general state of the art which is not considered to be of particular relevance	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
E earlier document published on or after the international filing date	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
L documents which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	*&* document member of the same patent family

Date of the actual completion of the international search

23 DECEMBER 1994

Date of mailing of the international search report

JAN 23 1995

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

JAN M. LUDLOW

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/11430

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

73/61.53; 204/180.1, 183.1-3, 299R, 302; 210/198.2; 422/59, 60, 70, 101; 436/161, 527, 531, 534